Environ. Sci. Technol. **2003**, *37,* 1850−1858

Inhibition of Bacterial U(VI) Reduction by Calcium

SCOTT C. BROOKS,*,† JAMES K. FREDRICKSON,‡ SUE L. CARROLL,† DAVID W. KENNEDY,‡ JOHN M. ZACHARA,‡ ANDREW E. PLYMALE,‡ SHELLY D. KELLY,§ KENNETH M. KEMNER,§ AND SCOTT FENDORF||

Environmental Sciences Division, P.O. Box 2008, MS 6038, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6038, Pacific Northwest National Laboratory, P.O. Box 999, Richland, Washington 99338, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439-4843, and Department of Geological and Environmental Sciences, Stanford University, Stanford, California 94305-2115

The rapid kinetics of bacterial U(VI) reduction and low solubility of uraninite ($UO_{2,cr}$) make this process an attractive option for removing uranium from groundwater. Nevertheless, conditions that may promote or inhibit U(VI) reduction are not well-defined. Recent descriptions of Ca−UO_2−CO_3 complexes indicate that these species may dominate the aqueous speciation of U(VI) in many environments. We monitored the bacterial reduction of U(VI) in bicarbonate-buffered solution in the presence and absence of Ca. XAFS measurements confirmed the presence of a Ca−U(VI)−CO_3 complex in the initial solutions containing calcium. Calcium, at millimolar concentrations (0.45−5 mM), caused a significant decrease in the rate and extent of bacterial U(VI) reduction. Both facultative (*Shewanella putrefaciens* strain CN32) and obligate (*Desulfovibrio desulfuricans*, *Geobacter sulfurreducens*) anaerobic bacteria were affected by the presence of calcium. Reduction of U(VI) ceased when the calculated system E_h reached −0.046 ± 0.001 V, based on the $Ca_2UO_2(CO_3)_3 \rightarrow UO_{2,cr}$ couple. The results are consistent with the hypothesis that U is a less energetically favorable electron acceptor when the Ca−UO_2−CO_3 complexes are present. The results do not support Ca inhibition caused by direct interactions with the cells or with the electron donor as the reduction of fumarate or $Tc(VII)O_4^-$ under identical conditions was unaffected by the presence of Ca.

Introduction

Dissimilatory metal-reducing bacteria (DMRB) couple the oxidation of organic matter or H_2 to the reduction of oxidized metals. Bacterial respiration based on the reduction of Fe(III) and Mn(III/IV) is an important process in the cycling of carbon and these metals in the environment (*1*−*3*). In recent years, a significant body of work has shown that DMRB can also reduce a number of toxic metals and radionuclides of environmental concern including Co(III)−EDTA, Cr(VI), Tc(VII), and U(VI) (*4*−*12*). From the standpoint of remediation of contaminated environments, the bacterial reduction of these metals is desirable as the lower oxidation states are less stable (Co−EDTA), less mobile, and have a lower solubility than when present at higher oxidation states.

Activities associated with the mining and processing of uranium (U) ores as well as defense-related activities have resulted in vast areas of contaminated soils and groundwater. Oxidized uranium (U(VI)) is much more soluble than the reduced form (U(IV)) and typically exists in groundwater as uranyl carbonate complexes. U(VI) is readily reduced by DMRB under anoxic conditions, resulting in the precipitation of uraninite ($U(IV)O_{2,cr}$) (*4*, *5*). The rapid rate of U(VI) reduction and the low solubility of U(IV) makes bioremediation an attractive option for removing U from contaminated groundwaters.

Despite the promise of bioreduction as a remediation strategy, the factors that may enhance or inhibit bacterial U(VI) reduction under environmental conditions are not well-defined. Previous research has shown that microbial reduction of U is difficult to predict and that results obtained under a particular set of conditions or by a particular microorganism are not necessarily transferable to other conditions or organisms. For example, the rate of U(VI) reduction by *Shewanella algae* strain BrY increased as U(VI) was complexed by multidentate organic ligands while U complexation by the same ligands decreased the rate of U(VI) reduction by *Desulfovibrio desulfuricans* (*13*). Phillips et al. (*14*) reported that U(VI) reduction by *D. desulfuricans* decreased as the concentration of bicarbonate was increased from 30 to 100 mM. The rate and extent of U(VI) removal from solution by *D. desulfuricans* decreased in the presence of sulfate (SO_4^{2-}) or nitrate (NO_3^-) at concentrations of 104 and 806 mM, respectively (*15*). The bacterial removal of U(VI) from water is also inhibited by the presence of competitive electron acceptors, such as iron(III) (hydr)oxides (*16*) and also inhibited by the presence of geochemical oxidants, such as manganese(IV) oxides (*17*, *18*).

Most equilibrium speciation models predict that the dominant U(VI) aqueous species in groundwater will be uranyl carbonate complexes (*19*, *20*). Nevertheless, Ca−U−CO_3 complexes ($CaUO_2(CO_3)_3^{2-}$, $Ca_2UO_2(CO_3)_3$) have recently been described (*21*−*23*) that have generally not been included in the speciation calculations. The magnitude of the formation constants for these species ($\log \beta_{113} = 25.4$; $\log \beta_{213} = 30.55$) suggests that they should be important aqueous species in many natural and contaminated environmental settings. For example, Abdelouas et al. (*20*) calculated the U(VI) species distribution in groundwater from the Tuba City, AZ, Uranium Mill Tailing Remedial Action (UMTRA) site. They reported that the species $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$ account for 56% and 38%, respectively, of the aqueous U(VI). Using the reported water composition and recalculating the species distribution including the Ca−U(VI)−CO_3 species indicates that $Ca_2UO_2(CO_3)_{3,aq}$ and $CaUO_2(CO_3)_3^{2-}$ account for 99.3% and 0.3%, respectively, of the U(VI) whereas $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$ combined account for less than 0.4% of the uranium. The $Ca_2UO_2(CO_3)_3$ complex may play an important role in the environmental chemistry of U. Its potential impact on bacterial U(VI) reduction has not been addressed to date. This paper reports on the systematic investigation of the influence of Ca on the bacterial reduction of U(VI).

* Corresponding author telephone: (865)574-6398; fax: (865)576-8646; e-mail: brookssc@ornl.gov.
† Oak Ridge National Laboratory.
‡ Pacific Northwest National Laboratory.
§ Argonne National Laboratory.
|| Standord University.

10.1021/es0210042 CCC: $25.00
Published on Web 04/02/2003

Materials and Analytical Methods

Cell Culturing and Harvesting. One facultative bacterium (*Shewanella putrefaciens* strain CN32) and two strict anaerobes (*Desulfovibrio desulfuricans* and *Geobacter sulfurreducens*) were used to evaluate the effects of Ca on bacterial U reduction. All of these bacteria were isolated from anaerobic sediments and are effective at enzymatically reducing U(VI) to U(IV). *S. putrefaciens* strain CN32 was provided courtesy of Dr. David Boone (Subsurface Microbial Culture Collection, Portland State University, Portland, OR). Strain CN32 was isolated from a subsurface core sample (250 m beneath the surface) from the Morrison Formation in northwestern New Mexico (*24*). CN32 was routinely cultured aerobically in tryptic soy broth (TSB), 30 g/L (Difco Laboratories, Detroit, MI), and stock cultures were maintained by freezing in 40% glycerol at −80 °C.

S. putrefaciens CN32 was grown aerobically in 250-mL Erlenmeyer flasks with 100 mL of TSB. Cultures were incubated for 16 h on a rotary shaker (100 rpm) at 30 °C. Cells were harvested by centrifugation (6000*g*, 15 min, 4 °C), washed once in 30 mM PIPES buffer (pH 7) and once with 30 mM sodium bicarbonate, and resuspended in 60 mL of 30 mM sodium bicarbonate that was made anoxic by purging with N_2:CO_2 (80:20). Cells were resuspended in a volume sufficient to achieve a concentration of about $2-4 \times 10^9$ cells/mL. For comparative purposes, *S. putrefaciens* CN32 was also grown anaerobically in a chemically defined medium (NB basal) (*25*) with 40 mM fumarate as the electron acceptor and 20 mM sodium lactate as the electron donor. Cells were harvested and washed as described above for aerobically grown cultures. Because of the lower biomass yield from these anaerobic cultures, only 5×10^7 cells/mL were used for the final concentration. Cells were stored on ice and used within 4 h.

D. desulfuricans was grown anaerobically in 100 mL Modified Starkey's Medium C (ATCC Medium 207) with sodium sulfate substituted for ferrous ammonium sulfate and an N_2 headspace. Cultures were incubated for 48 h on a rotary shaker (100 rpm) at 30°C. Cells were harvested by centrifugation and washed in anaerobic PIPES and bicarbonate buffers as described above. Cells were resuspended in 30 mM sodium bicarbonate to $5-8 \times 10^8$ cells/mL and diluted to a final concentration of $(5-8) \times 10^7$ cells/mL.

G. sulfurreducens was grown in a chemically defined medium (NB basal) (*25*) with 40 mM fumarate as the terminal electron acceptor and 20 mM sodium acetate as the electron donor. Cultures were incubated for 48 h on a rotary shaker (100 rpm) at 30 °C. Cells were harvested as described above and resuspended at 9×10^7 cells/mL final concentration.

Bacterial U(VI) and Tc(VII) Reduction. Reaction vessels for experiments were either 25-mL pressure tubes or 60-mL serum bottles. The reduction of U(VI), from uranyl acetate, by metal-reducing bacteria was evaluated in the presence and absence of calcium. The base solution consisted of anaerobic 30 mM $NaHCO_3$ under a headspace of N_2:CO_2 (80:20), pH 6.9. The electron donors lactate (from either sodium lactate or $Ca(lactate)_2$) or acetate (sodium acetate) were added from stock solutions to achieve final concentrations of 5 mM. The effect of electron donor was evaluated in select experiments by adding 10 mL of H_2 gas to the headspace in place of lactate. Calcium concentrations were varied via the addition of $CaCl_2$ or $Ca(lactate)_2$. All reduction studies were conducted at 30 °C with gyratory shaking.

Tc(VII) Reduction. *S. putrefaciens* CN32 cells were cultured and harvested as described above and resuspended at a final concentration of $2-4 \times 10^7$/mL in 30 mM $NaHCO_3$ under a headspace of N_2:CO_2 (80:20) to obtain a final pH of 6.9. $NH_4Tc(VII)O_4$ (Amersham Life Sciences Products, Arlington Heights, IL) was added to approximately 50 μM.

Analyses. At selected time points, samples were taken in an anaerobic glovebag using needles and syringes. The reduction of U was determined by measuring the loss of U(VI) from solution using a kinetic phosphorescence analyzer (Chemcheck, Ins., Richland, WA) as described previously (*5*). The reduction of technetium was evaluated by direct extraction and liquid scintillation counting of ^{99}Tc (0.292 MeV β) (*11*). Briefly, in an anaerobic glovebox, the filtered (0.2 μm) sample was added to tetraphenyl arsonium chloride (*26*) to obtain a TPAC:Tc molar ratio of 40:1. Samples were removed from the glovebox, weighed, and extracted with chloroform. The resulting chloroform layer was removed, and the concentration of Tc was determined by liquid scintillation counting. Lactate, fumarate, and succinate were quantified by capillary electrophoresis using a Waters Quanta 4000E instrument and direct detection at 185 nm. Run buffer was 25 mM $Na_2B_4O_7$, 0.6 mM $CaCl_2$, and 0.5 mM tetradecyltrimethylammonium hydroxide (TTAOH), pH 9.2. Capillary: 75 μm i.d. × 55 cm to the detector (62 cm total length). Separation conditions: 25 °C, −15 kV constant voltage.

XAFS Analysis. Fluorescence XAFS measurements of the base solution (50 μM U, 5 mM $CaCl_2$, and 30 mM HCO_3 under anaerobic conditions with a 80:20 N_2:CO_2 ratio headspace) at the U L_3 absorption edge (17166 eV) were performed at the Materials Research Collaborative Access Team (MR-CAT) (*27*) beamline 10-ID at the Advanced Photon Source at Argonne National Laboratory. Beamline parameters were as follows: Incident X-ray energy was selected by using a double-crystal Si(111) monochromator. The third harmonic of the undulator was tapered 3.5 keV to reduce the variation of the incident intensity to less than 20% over the scanned energy range (~1000 eV). A Rh mirror rejected X-rays of higher harmonic energies. Incident X-ray intensity was monitored with a N_2-filled ion chamber. Fluorescent X-ray intensity was monitored with a 13-element solid-state detector (Canberra with X1A electronics). EXAFS data were analyzed with the codes contained in the UWXAFS package (*28*) and IFEFFIT (*29*). Data collected from 11 elements of the solid-state detector for each energy scan were averaged to produce 10 absorption spectra. Background was removed from each of these data sets with the ATHENA program (*30*), and the resulting χ(k) data were averaged. Theoretical models were constructed with the program FEFF7 (*31*) and crystallographic atomic positions of andersonite (*32*). Models were fit to the data by using the fitting routine FEFFIT (*33*), which also performs error analysis and calculates the goodness-of-fit parameters. Details on the fitting procedure have been described previously for similar systems (*34*, *35*).

Results

Aqueous Speciation of U(VI) in Initial Solutions. *Equilibrium Calculations.* The rate and extent of U(VI) removal from solution by the various DMRB was monitored as a function of calcium concentration. The U aqueous species distribution in the various solutions prior to bacterial reduction was calculated using the React module in the commercially available software program The Geochemists Workbench (*36*). Formation constants for the U(VI) complexes were obtained from the extensive compilation of Grenthe et al. (*37*). Formation constants for uranium(VI) acetate complexes and $Ca-UO_2-CO_3$ complexes were from Shock and Koretsky (*38*) and Bernhard et al. (*23*), respectively. In the absence of Ca, $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$ are predicted as the dominant aqueous species but decrease in abundance as the concentration of Ca in solution increases with a concomitant increase in $Ca_2UO_2(CO_3)_3$ (Table 1). The predicted percent distribution of aqueous U(VI) species is invariant as the U(VI) concentration is decreased.

XAFS Measurements. The average $\chi(k)k$ data are shown in Figure 1a. The magnitude of the Fourier transform (FT) of

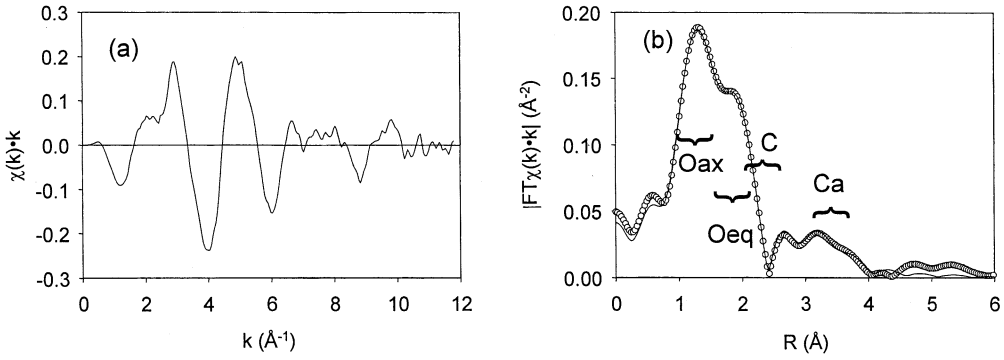

FIGURE 1. (a) XAFS $\chi(k)\cdot k$ data for Ca-containing base solution. (b) Magnitude of the Fourier transform of the data shown in panel a (open circles) and best-fit model (thick line). Data processed with $\Delta k = 3.3-9.3$ Å^{-1}, $\Delta R = 0.9-4.0$ Å, and a Hanning window with a full sill width of 1.0 Å^{-1}. O_{ax}, axial oxygen; O_{eq}, equatorial oxygen.

TABLE 1. U(VI) and Calcium Aqueous Species Distribution for the Conditions in These Experiments[a]

aqueous species	no Ca	0.45 mM Ca	2.5 mM Ca	5.0 mM Ca
		Mol % Total U(VI)		
$UO_2(CO_3)_3^{4-}$	76.8	34.4	2.2	0.7
$UO_2(CO_3)_2^{2-}$	23.1	10.2	0.6	0.2
$Ca_2UO_2(CO_3)_3$		51.0	95.8	98.4
$CaUO_2(CO_3)_3^{2-}$		4.3	1.4	0.8
		Mol % Total Ca		
Ca^{2+}		71.0	77.7	80.0
$CaHCO_3^+$		11.9	12.5	12.3
calcium lactate$^+$		4.7	4.9	4.8
$CaCO_{3,aq}$		0.7	0.7	0.7
$Ca_2UO_2(CO_3)_3$		11.3	3.8	2.0
$CaUO_2(CO_3)_3^{2-}$		0.5	0.03	

[a] Base medium consisted of 30 mM $NaHCO_3$, 5 mM sodium lactate, 50 μM UO_2(acetate)$_2$, and 20% CO_2 at pH 6.9.

TABLE 2. Best-Fit Values for XAFS Model of Ca-Containing Base Solution[a]

scattering path[b]	N_{degen}[c]	R (Å)[d]	σ^2 (10^{-3} Å^2)[e]	ΔE_0 (eV)[f]
U−O_{ax}	2	1.78	1 ± 1	0.2 ± 0.5
U−O_{eq}	5.5 ± 0.7[g]	2.45 ± 0.01	6 ± 2	6.8 ± 1.0
U−C	2.7 ± 0.4[g]	2.90	4 ± 2	6.8 ± 1.0
U−O_{ax1}−O_{ax2}	2	3.56	2 ± 2	0.2 ± 0.5
U−O_{ax1}−U−O_{ax1}	2	3.56	2 ± 2	0.2 ± 0.5
U−O_{ax1}−U−O_{ax2}	2	3.56	2 ± 2	0.2 ± 0.5
U−Ca	3.4 ± 0.9	4.01 ± 0.01	6 ± 3	6.8 ± 1.0

[a] Values without uncertainties were constrained to the value listed. [b] Scattering path of the photoelectron. Single scattering paths are denoted U−X where X is the type of atoms in a shell about the uranium atoms. O_{ax}, axial oxygen; O_{eq}, equatorial oxygen. [c] Degeneracy of the photoelectron scattering path. For a single scattering path, this is the number of atoms in a shell about the uranium atoms. [d] The half path length of the photoelectron scattering path. For a single scattering path, this is the distance from the uranium atoms to a shell of atoms. [e] Mean-square displacement of R(Å). For a single scattering path, this represents the amount of structural and thermal disorder in the shell of atoms about the uranium atoms in the sample. [f] Energy shift of the photoelectron scattering path. [g] Number of O_{eq} and C atoms were determined based on a single variable for the number of CO_3 groups.

the EXAFS data and model are shown in Figure 1b. Contributions to XAFS signal used to model these data include (i) two tightly bound axial oxygen atoms, (ii) six equatorial oxygen atoms, (iii) three carbon atoms (which are part of the CO_3 groups bound to the uranyl moiety), and (iv) ~three calcium atoms (bound to the carbonates). Additional details for this model are contained in Table 2. Results of the fitting of the k dependence of the backscattering amplitudes in the region of ~4 Å in the FT are consistent with the presence of a Ca backscattering atom and are not consistent with a C or O backscattering atom. Therefore, the contribution of multiple scattering processes to the distal O atoms of the carbonate groups are not included in our fits, as described in a previous study (*23*). The number of atoms in each coordination shell, their distance to the absorbing uranium atom, and their mean-square displacement values are similar to those found for other uranyl coordination environments (*23*, *34*, *39*). Results of this fitting are in good agreement with the XAFS data and consistent with the formation of a Ca−UO_2−CO_3 complex, thus confirming the presence of this type of moiety in the base solutions.

U(VI) Reduction by *S. putrefaciens* CN32. Uranyl (U(VI)) reduction by aerobically cultured *S. putrefaciens* CN32 (CN32) with lactate as electron donor proceeded rapidly with complete reduction within 30 h (Figure 2a). There was no lag phase prior to U(VI) reduction for this treatment. The addition of 0.45 mM Ca to the medium resulted in a 5-h lag phase and a marked decrease in the rate and extent of U reduction with only ~75% of the initial U(VI) reduced after 97 h. Further increasing the concentration of Ca to 2.5 mM extended the lag phase to 60 h before the onset of U reduction. Reduction of U(VI) proceeded much more slowly once initiated, and only 70% of U(VI) was reduced after about 6 days with little or no additional reduction over the ensuing 4 days even though excess lactate (>4 mM) remained in solution (Figure 2a).

Possible causes for the inhibition of U(VI) reduction in the presence of Ca include (i) the complexation of lactate by Ca making it less bioavailable as an electron donor, (ii) a direct physiological effect of Ca on the cells that inhibited U(VI) reduction, or (iii) the formation of an aqueous Ca−U−CO_3 complex that was less susceptible to enzymatic reduction by CN32. The experiments described below address each of these possible causes.

Equilibrium speciation calculations indicated that 95% of the lactate remained as free lactate with 2.5 mM Ca present, suggesting that formation of calcium lactate complexes was not the reason for the observed effect. Nevertheless, this possible cause was evaluated directly in reduction experiments using anaerobically grown (with fumarate) CN32 cells using H_2 as the electron donor. The rate of U reduction with H_2 as electron donor was comparable to that observed for lactate in the absence of Ca (Figure 2b). There was no lag phase, and U(VI) reduction was complete within 13 h. These results are consistent with an earlier report that H_2 supports a comparable but slightly faster rate of U(VI) reduction by CN32 (*40*). The addition of 0.45 mM Ca (as $CaCl_2$) to the medium resulted in a decrease in the rate of U reduction. When the concentration of Ca was 5 mM, there was a substantial lag phase prior to the onset of U reduction. Only 37% of U was reduced after 72 h with no additional U(VI) loss

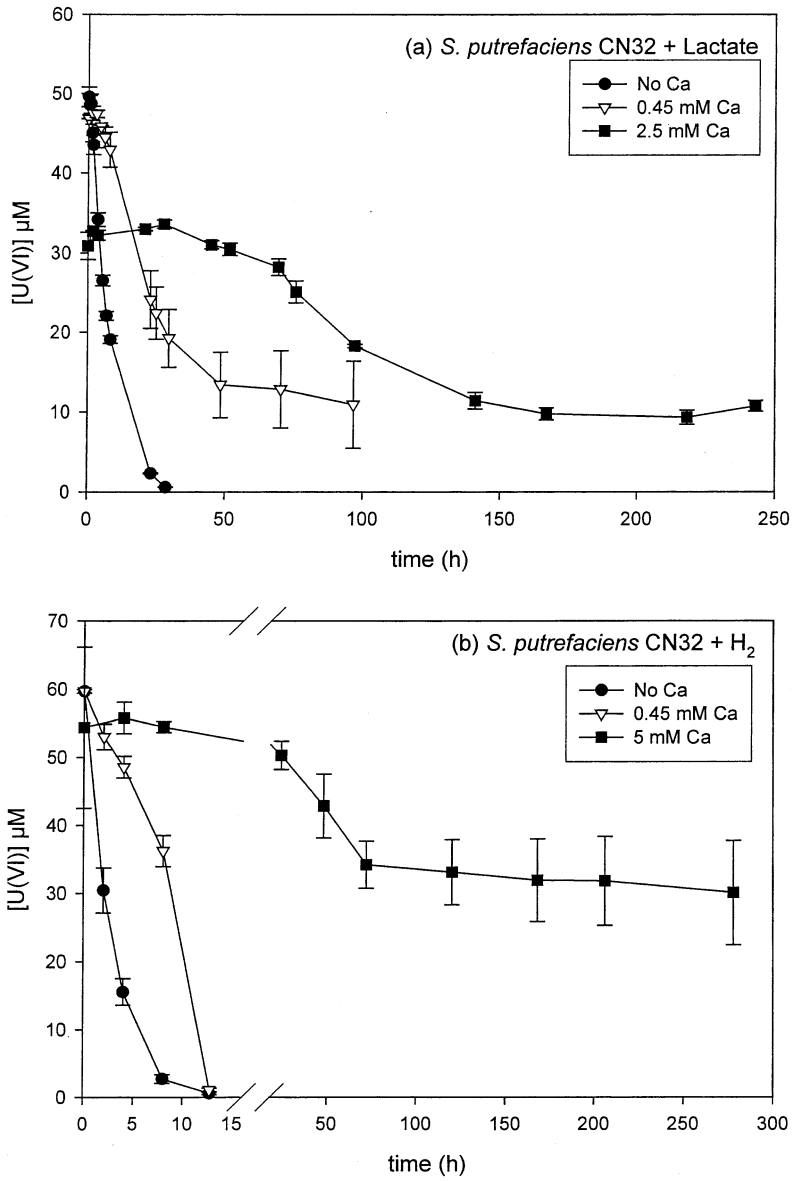

FIGURE 2. Removal of U(VI) from solution by *S. putrefaciens* strain CN32 at varying concentrations of Ca using either (a) aerobically cultured cells and lactate as electron donor or (b) anaerobically cultured cells and H_2 as electron donor.

over the ensuing 8.6 days (Figure 2b). Thus, the inhibition caused by Ca was independent of the electron donor, suggesting either specific Ca-cell or Ca−U interactions as the cause of the observed effect.

An alternate electron acceptor, fumarate, was selected to investigate whether exposure to the Ca−U(VI)−CO_3 complex or the length of experiments adversely impacted CN32. The reduction potential of the fumarate→succinate couple under the experimental conditions is similar to that of the uranyl carbonate species (Table 3). A U(VI) reduction experiment was conducted as before except the final cell concentration was $\sim 5 \times 10^7$ cells/mL as compared to $\sim 10^8$ cells/mL in other experiments. The effect of Ca on U(VI) reduction was comparable to that observed previously (Figure 3). On the sixth day, fumarate from a concentrated stock solution was added to the reaction vessels to achieve an initial concentration of $\sim 500\,\mu$M. The dilution effect from adding the fumarate was less than 3%. The loss of fumarate and stoichiometric production of succinate was monitored over time. Fumarate consumption proceeded rapidly with greater than 95% loss in less than 2 h, regardless of the presence of Ca (Figure 3). The results of this experiment suggest that neither length of the incubations nor exposure to the Ca−U(VI)−CO_3 complex negatively impacted the ability of CN32 to reduce fumarate.

Another alternate electron acceptor, Tc(VII), was chosen to investigate whether Ca−cell interactions or Ca effects on organism physiology may be responsible for a general inhibition of metal reduction. The loss of Tc from solution was monitored in suspensions of CN32 using either 5 mM sodium lactate or 2.5 mM Ca(lactate)$_2$ as electron donor. The presence of Ca in the medium did not inhibit the loss of Tc from solution (Figure 4). In fact, Tc(VII)(aq) loss proceeded more rapidly in the presence of 2.5 mM Ca, although additional experiments are needed to establish whether this effect is significant. Nevertheless, the results of this experiment suggest that Ca per se does not have a direct physiological effect on cells of strain CN32. In addition, these results lend further support to the previous results showing that formation of calcium lactate complexes did not inhibit metal reduction.

Similar to the results reported for Tc, Zhang et al. (*41*) reported rapid U(VI) reduction with no evidence of a lag phase by another metal-reducing *Shewanella* strain, *S. algae* BrY, using H_2 as an electron donor in carbonate free medium

TABLE 3. Half-Cell Potentials of Aqueous U(VI) Species and Electron Donors Used in the Experiments

		E° (V)	E (V)[a]
	Electron Acceptor		
(1)	$UO_2^{2+} + 2e^- \rightarrow UO_{2,cr}$ (uraninite)	0.411	0.284[b]
(2)	$UO_2(CO_3)_3^{4-} + 3H^+ + 2e^- \rightarrow UO_{2,cr} + 3HCO_3^-$	0.689	0.086[b]
(3)	$UO_2(CO_3)_2^{2-} + 2H^+ + 2e^- \rightarrow UO_{2,cr} + 2HCO_3^-$	0.521	0.077[b]
(4)	$Ca_2UO_2(CO_3)_{3,aq} + 3H^+ + 2e^- \rightarrow UO_{2,cr} + 2Ca^{2+} + 3HCO_3^-$	0.424	−0.042[c]
(5)	$CaUO_2(CO_3)_3^{2-} + 3H^+ + 2e^- \rightarrow UO_{2,cr} + Ca^{2+} + 3HCO_3^-$	0.576	0.042[c]
(6)	$C_4H_4O_4^{2-} + 2H^+ + 2e^- \rightarrow C_4H_4O_4^{2-}$ (fumarate) (succinate)	0.407	0.079[d]
(7)	$^2/_3TcO_4^- + 2e^- + {}^8/_3H^+ \rightarrow {}^2/_3TcO_2 + {}^4/_3H_2O$	0.747	0.118[e]
	Electron Donor		
(8)	$H^+ + e^- \rightarrow 1/2\ H_2(g)$	0	−0.408[f]
(9)	$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O$ (acetate)	0.187	−0.278[g]
(10)	$3HCO_3^- + 14H^+ + 12e^- \rightarrow C_3H_5O_3^- + 6H_2O$ (lactate)	0.156	−0.332[h]
(11)	$CH_3COO^- + HCO_3^- + 4e^- + 5H^+ \rightarrow C_3H_5O^- + 2H_2O$	0.093	−0.465[i]

[a] Reduction potential under experimental conditions. [b] pH 6.9; HCO_3^-, 28.7 mM (calculated concentration under the 20% CO_2 headspace); U(VI), 50 μM; Ca, 5 mM. [c] HCO_3^-, 28.1 mM; Ca, 5 mM; other conditions as in footnote *b*. [d] Fumarate, 0.5 mM; succinate, 0.001 mM. [e] TcO_2, hydrous oxide based on observations of Wildung et al. (*11*); Tc data from Lemire and Jobe (*50*). [f] $P_{H_2(g)} = 1$ atm. [g] pH 6.9; HCO_3^-, 28.7 mM; CH_3COO^-, 5 mM. [h] pH 6.9; HCO_3^-, 28.7 mM; $C_3H_5O_3^-$, 5 mM. [i] *S. putrefaciens* CN32 incompletely oxidizes lactate to acetate and CO_2. pH 6.9; HCO_3^-, 28.7 mM; CH_3COO^-, 0.1 mM from UO_2(acetate)$_2$ used as the source of U(VI); $C_3H_5O_3^-$, 5 mM.

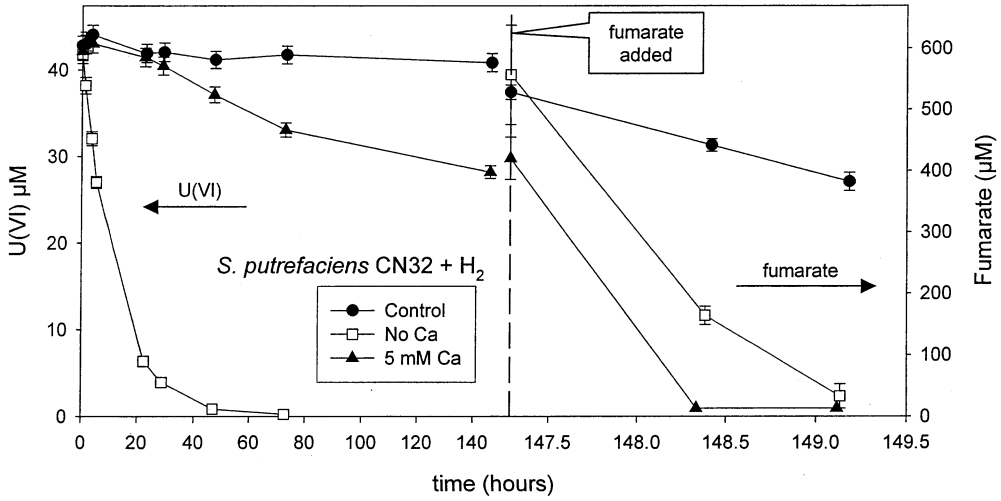

FIGURE 3. Reduction of U(VI) and fumarate by aerobically cultured cells of *S. putrefaciens* strain CN32 (~5 × 10^7 cells/mL) using H_2 as electron donor. Fumarate was added to media after 6-day incubation.

containing 5 mM $CaCl_2$ at pH 7. These results suggest that a direct Ca−cell interaction was not responsible for the observed inhibition of U reduction, although, as noted in the Introduction, comparison between strains can be problematic. However, Ca may have an indirect effect on enzymatic reduction of U, possibly via the formation of a $Ca_2UO_2(CO_3)_3$ aqueous complex that may be of lower biological availability.

U(VI) Reduction by Obligate Anaerobic Bacteria. The results described above demonstrate that Ca inhibits the rate and extent of U(VI) reduction by *S. putrefaciens* CN32 in the presence of CO_2/HCO_3, putatively through the formation of a Ca−U−CO_3 complex. Given the diversity of bacteria that can reduce U(VI), we sought to establish whether this effect was specific to the facultative anaerobe CN32 or was more generally applicable to other uranium-reducing bacteria. We therefore investigated whether U(VI) reduction by the obligate anaerobes *D. desulfuricans* and *G. sulfurreducens* was inhibited by Ca.

The loss of U(VI) from solution with *D. desulfuricans* was monitored over time in the absence and presence of 5 mM Ca. U(VI) loss from solution was rapid in the absence of Ca using either lactate or H_2 as electron donor (Figure 5). Virtually all the U(VI) was removed from solution within 5 days. There was a marked decrease in the rate and extent of U reduction when 5 mM Ca was included in the medium, regardless of electron donor. Less than 20% U(VI) was removed from solution over 5 days when Ca was present.

G. sulfurreducens reduced 35% U(VI) within 48 h with 5 mM acetate as the electron donor in the absence of Ca. No further reduction was observed over an additional 10 days of incubation (Figure 6). The low acetate control replicates shown in Figure 6 had 0.1 mM acetate that was associated with the UO_2(acetate)$_2$ used as the source of U(VI). Given the experimental variation, U(VI) reduction at the two concentrations of acetate were not significantly different. Incubations that included 5 mM Ca showed no U(VI) loss over the first 5 days with less than 20% loss over the subsequent 7 days.

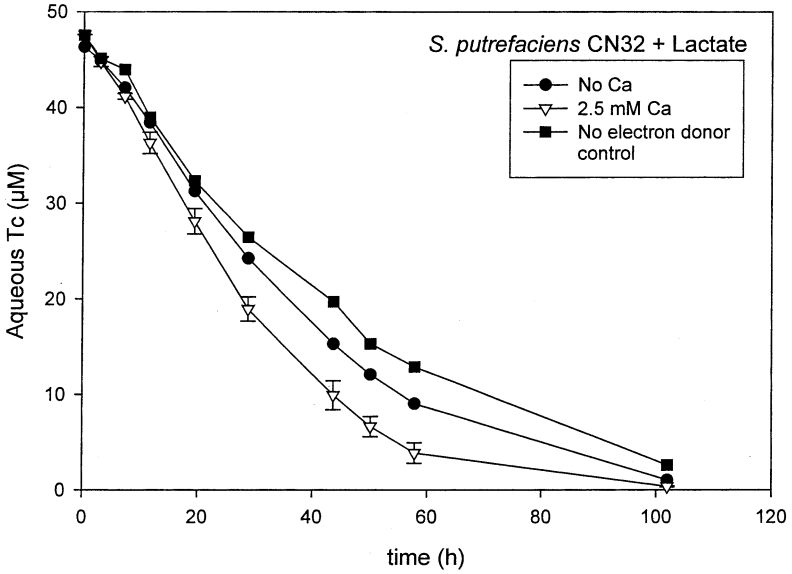

FIGURE 4. Removal of Tc from solution by *S. putrefaciens* strain CN32 in the absence and presence of Ca. Loss of Tc in the control experiments is the result of endogenous respiration by fresh cultures of CN32 grown in TSB.

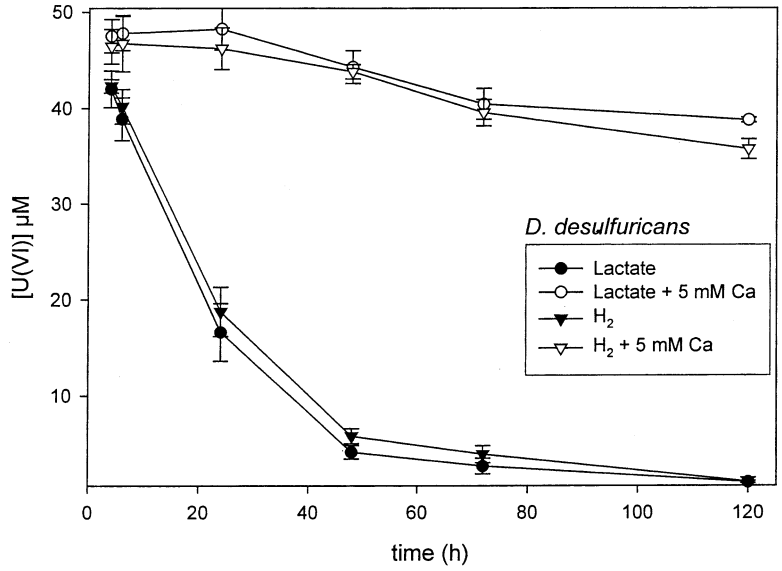

FIGURE 5. U(VI) reduction in bicarbonate-buffered medium by *D. desulfuricans* using either lactate or H_2 as electron donor.

The reason for the relatively poor reduction of U by *G. sulfurreducens* in the absence of Ca is unclear, although the initial report describing the isolation and characterization of strain PCA indicated that it was unable to utilize U(VI) or Mn(IV) as electron acceptors (*42*).

Various media have been used in batch metal reduction experiments reported in the literature. In some cases, trace metals or vitamins are added to the solution to enhance cell maintenance and/or growth (*16, 43*). Including these amendments in the medium may complicate interpretation of results as the trace metal and vitamin solutions both contain redox active components that can potentially act as electron-shuttling compounds promoting the reduction of the electron acceptors of interest. For example, the DMRB *S. algae* strain BrY cannot effectively reduce carbon tetrachloride (CCl_4) or chloroform (CF) via direct mechanisms. BrY does, however, reduce vitamin B_{12} that can subsequently reduce CCl_4 and CF (*44*).

The effect of trace mineral and vitamin amendments on U reduction was tested in a series of experiments with CN32 using lactate as electron donor and 2.5 mM Ca. Trace mineral and vitamin solutions were the same as described by other investigators (*16, 25, 45*). The trace minerals solution contains NTA (final concentration, 0.0785 mM) that could alter U species distribution either directly through formation of U−NTA complexes or indirectly via complexation of Ca. Results of equilibrium speciation modeling indicated that the predicted initial concentration and distribution of U species was not affected by the trace minerals solution. Including trace minerals in the bicarbonate-buffered solution resulted in a substantially faster loss of U(VI) from solution (Figure 7). Nevertheless, there was still a lag phase (11 h) prior to the onset of U reduction, and reduction was incomplete (96%). There was no lag in reduction when trace vitamins were included in the solution, and the rate of U loss was comparable to when Ca was absent. However, U loss ceased after the removal of 84% U(VI). The addition of trace minerals and vitamins together also enhanced the rate of U loss from solution. There was still a notable lag phase (23 h), and U reduction was again incomplete (97%) after 6 days. The addition of both amendments yielded slower U loss relative to the addition of either the trace minerals or vitamins

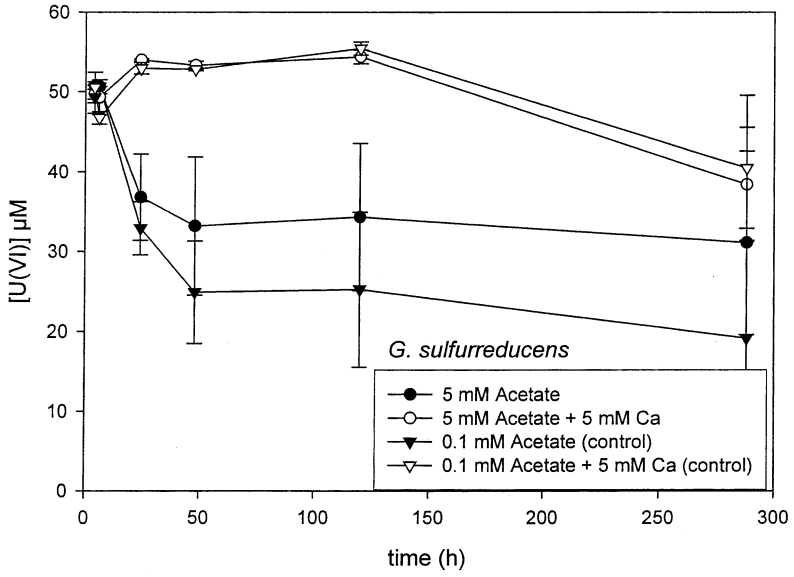

FIGURE 6. U(VI) reduction in bicarbonate-buffered medium by *Geobacter sulfurreducens* using acetate as electron donor.

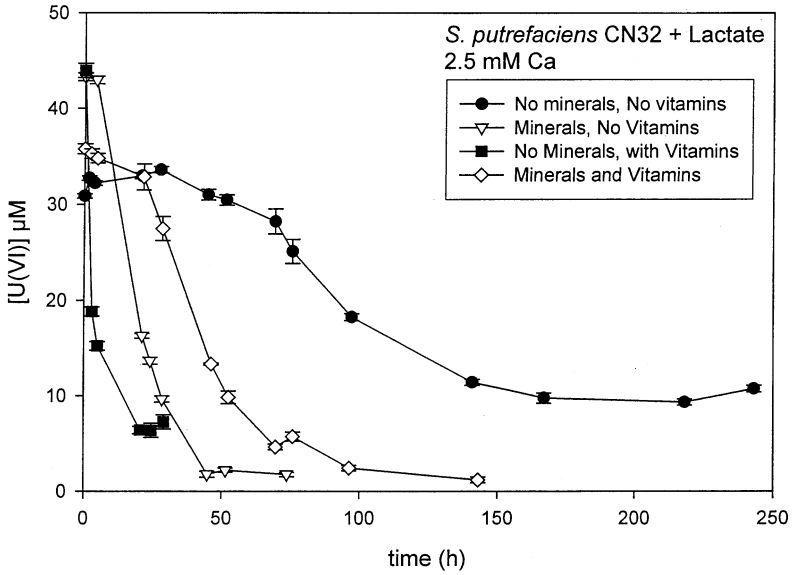

FIGURE 7. Effect of trace minerals and vitamins on U(VI) removal from solution by *S. putrefaciens* strain CN32 from bicarbonate-buffered medium with 2.5 mM Ca and lactate as electron donor.

separately. We observed no increase in cell numbers over the course of these experiments, although these amendments may have promoted metabolic reactions that promoted U(VI) reduction. Alternately, these results may be due to indirect effects such as electron shuttling by components of the trace minerals or vitamins solution.

Calcium, at millimolar concentrations, caused a significant decrease in the rate and extent of bacterial U(VI) reduction. Both facultative and obligate anaerobic U-reducing bacteria were affected by the presence of calcium. The results are consistent with the hypothesis that U is a less effective electron acceptor when the $Ca_2UO_2(CO_3)_3$ complex is present. The results do not support Ca inhibition caused by direct interactions with the cells or with the electron donor.

Complexation of uranium stabilizes the higher oxidation state, lowering the reduction potential of the U(VI) → $UO_{2,cr}$ couple. Equilibrium thermodynamic calculations indicate that $Ca_2UO_2(CO_3)_3$ is a weaker electron acceptor than the other U(VI) forms predicted to exist in the experiments with a reduction potential more than 0.1 V lower than $UO_2(CO_3)_3^{4-}$ (Table 3). Using the $Ca_2UO_2(CO_3)_3$ → $UO_{2,cr}$ couple, the final system E_h was calculated for the experiments with 5 mM Ca. Uranium(VI) reduction ceased when the system E_h reached −0.046 V (Table 4). Thus, the observed plateau in U(VI) reduction in the presence of 5 mM Ca may represent a fundamental limit on the extent of direct bacterial U(VI) reduction that can be effected by the terminal reductase enzymes and their measured or theoretical midpoint potentials in these bacterial strains. At present, the enzymes responsible for U reduction in these organisms are not well-characterized so it is not possible to directly compare the

TABLE 4. Computed Final E_h Based on the $Ca_2UO_2(CO_3)_3$ → $UO_{2,cr}$ Couple for Experiments Containing 5 mM Ca

organism	e⁻ donor	E_h (V)	Figure
S. putrefaciens CN32	H_2	−0.048	2b
D. desulfuricans	lactate	−0.045	5
D. desulfuricans	H_2	−0.046	5
G. sulfurreducens	acetate (5 mM)	−0.045	6
G. sulfurreducens	acetate (0.1 mM)	−0.044	6
	average ± SD	−0.046 ± 0.001	

calculated system E_h with the enzyme reduction potentials. However, cytochromes with low midpoint potentials have been described for *S. putrefaciens* (−0.233 V; *46*), *D. desulfuricans* (−0.165 to −0.4 V; *47*), and *G. sulfurreducens* (−0.167 V; *48*). It is not known if these cytochromes are involved in the direct reduction of U(VI) by these bacteria.

The uranium reduction potentials described above indicate the sequence in which U(VI) species will be used according to their thermodynamic possibility, but they do not make a prediction about the rate of reaction. Nor do the reduction potentials account for the lag phase observed. Even at the highest calcium concentrations tested, some U(VI) remains as $UO_2(CO_3)_3^{4-}$ and $UO_2(CO_3)_2^{2-}$ complexes (Table 1), which should be available for reduction by the bacteria. As these species are consumed, the system will reequilibrate, maintaining a pool of these species in solution. The slow rate of reduction in the presence of calcium may reflect a kinetically slow reequilibration of the aqueous U(VI) complexes representing the rate-determining step in the removal of U(VI) in these systems. Alternately, the $Ca_2UO_2(CO_3)_3$ complex may act as a competitive inhibitor of the reduction other U(VI) species (e.g., $UO_2(CO_3)_3^{4-}$) that is either reduced more slowly or not at all.

Calcium and dissolved CO_2 are ubiquitous components of natural and contaminated groundwater. In addition, the alkaline leaching of some U ores has introduced significant amounts of HCO_3^- into impacted groundwater (*49*). At the Field Research Center for the U.S. Department of Energy's Natural and Accelerated Bioremediation Research (NABIR) program located on the Oak Ridge site in eastern Tennessee, groundwater has been contaminated with uranium-bearing nitric acid wastes that percolated through carbonate host rock. The resulting calcium concentrations at the site range from 1 to 300 mM, and carbonate alkalinity ranges from 1 to 10 mM (http://public.ornl.gov/nabirfrc/dataarea123.cfm; accessed August 6, 2002). Similar concentrations of Ca and alkalinities are found at Uranium Mill Tailing Remedial Action (UMTRA) sites (*20*). The dominant U(VI) species in seepage water from U mine tailings in Germany is $Ca_2UO_2(CO_3)_3$ (*21*). Bacterially mediated U(VI) reduction is being explored or tested at both the NABIR site and UMTRA sites for the in situ removal of uranium from groundwater. Results of this research indicate that the inorganic $Ca_2UO_2(CO_3)_3$ complex may pose a fundamental limit for enzymatic reduction by DMRB that must be addressed prior to the successful application of bacterial U(VI) reduction for the purpose of groundwater cleanup.

Acknowledgments

This research was funded by the U.S. Department of Energy's Office of Science Biological and Environmental Research, Natural and Accelerated Bioremediation Research (NABIR) program. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the U.S. Department of Energy under Contract DE-AC05-00OR22725. Pacific Northwest National Laboratory is operated for the DOE by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830. The Advanced Photon Source is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences. Work performed at MRCAT (beamline 10-ID) is supported, in part, by funding from the Department of Energy (Grant DEFG0200ER45811). We thank E. J. O'Loughlin and J. H. Terry for assistance with XAFS measurements.

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Received for review October 28, 2002. Revised manuscript received February 25, 2003. Accepted February 26, 2003.

ES0210042